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**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

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Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**I.L.C. HATTEN-HECKMAN**

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**Blatt 2 der Bescheinigung  
Sheet 2 of the certificate  
Page 2 de l'attestation**

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**Methods of diagnosing or prognosing age-related macular degeneration**

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## METHODS OF DIAGNOSING OR PROGNOSING AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the most common geriatric eye disorder leading to blindness. Macular degeneration is responsible for visual handicap in what is estimated conservatively to be approximately 16 million individuals worldwide. Histopathologically, the hallmark of early neovascular AMD is accumulation of extracellular drusen and basal laminar deposit (abnormal material located between the plasma membrane and basal lamina of the retinal pigment epithellum) and basal linear deposit (material located between the basal lamina of the retinal pigment epithelium and the inner collagenous zone of Bruch's membrane). The end stage of AMD is characterized by a complete degeneration of the neurosensory retina and of the underlying retinal pigment epithelium in the macular area. The etiology of AMD is largely unknown, but the current understanding is that AMD is a genetically complex eye disorder possibly caused by a variety of molecular defects.

Klaver et al. (Am. J. Human Genet. 63: 200 – 206, 1998) have shown that the apoE gene (APOE) polymorphism is significantly associated with the risk for AMD. The APOE  $\epsilon$ 4 allele was associated with a decreased risk, and the  $\epsilon$ 2 allele was associated with a slightly increased risk of AMD. Their results suggest that APOE is a susceptibility gene for AMD.

Experimental therapies for AMD have been suggested by Soubrane and Coscas (Wiedemann P., Kohen L. (eds): Macular and retinal diseases. Dev. Ophthalmol. Basel, Karger vol. 29, 77 – 84, 1997). The goal of therapies can be twofold: (i) prevention of the occurrence of macular complications or (ii)

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treatment of the already arisen macular complications (central geography atrophy or choroidal neovascularization). One approach for prevention includes antioxidant supplementation with contradictory results regarding the effectiveness. Laser photocoagulation has also been largely ineffective in preventing visual loss in the majority of patients. However, in the therapy of macular complications, laser photocoagulation remains the gold standard for the 15 % of well-defined choroidal new vessels (CNVs). For isolated occult CNVs and vascularized pigment epithelium detachments, no effective treatment is at the horizon. For geographic atrophy the only hope is at present retinal pigment epithelium and photoreceptors transplantation.

As AMD is a growing social and medical problem, there is a strong need for methods of diagnosing or prognosing said disease in subjects as well as for methods of treatment. In addition, there is a strong need for identifying inherited risk factors that increase the susceptibility of getting AMD (susceptibility gene).

Several complex diseases previously associated with aging and exposure to environmental risk factors have now been found to have a strong genetic component. Late onset Alzheimers disease (AD) is an example for these conditions. It is noteworthy that AD shares the same epidemiology with AMD, and both diseases have similarities with respect to their pathophysiology. Like AD, in a broad sense AMD can be considered to be a neurodegenerative disorder. Deposits are found in both conditions and during the natural course of these diseases a significant loss of neurons occurs.

In one aspect, the invention features a method for diagnosing or prognosing age-related macular degeneration in a subject, or determining whether a subject is at increased risk of becoming diseased with age-related macular degeneration. The method includes: determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the

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group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for said amyloid protein in a sample from said subject; and comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status, thereby diagnosing or prognosing said age-related macular degeneration in said subject, or determining whether said subject is at increased risk of becoming diseased with age-related macular degeneration.

The human cystatin C gene (*CST3*) maps to chromosome 20p11.2, it contains three exons, and a *Sst* II polymorphic site within the 5' flanking sequence that is in linkage disequilibrium with a second *Sst* II polymorphism within exon 1 of the gene (Balbin and Abrahamson, *Hum. Genet.* 81, 751 - 752, 1991; Balbin et al., *Hum. Genet.* 92, 206 - 207, 1993; Abrahamson et al., *FEBS Lett.* 216, 229 - 233, 1987). The open reading frame of *CST3* encodes a 120-residue protein with a molecular mass of 13.3 kDa and a pI of 8.75 (Abrahamson et al., *J. Biol. Chem.* 261, 11282 - 11289, 1986; Abrahamson et al., *FEBS Lett.* 216, 229 - 233, 1987). The mature molecule contains intramolecular disulfide bonds, it is partially hydroxylated, no other common post-translational modifications were observed (Grubb and Löfberg, *Proc. Natl. Acad. Sci., USA*, 79, 3024 - 3027, 1982; Asgelsson et al., *Biochem. J.*, 329, 497 - 503, 1998). Cystatin C is distributed extensively in the body fluids and is suspected of playing a role in extracellular functions, such as the modulation of inflammatory reactions. It is known to exist in cell types, such as astrocytes, macrophages, and choroid plexus cells. Cystatin C also is a quantitatively dominating cysteine protease inhibitor of CSF whose concentration is five times higher than that of plasma. It binds to and regulates proteolytic activities of cathepsins which have been associated with brain amyloid plaques in Alzheimer's disease, and implicated in the proteolytic processing of the amyloid precursor protein. It has been described that human cystatin C undergoes dimerization before unfolding.

Dimerization leads to a complete loss of its activity as a cysteine proteinase inhibitor (Ekiel et al., J. Mol. Biol. 271, 266 - 277, 1997). In addition to the termination of biological activity, dimerization of cystatin C is associated with brain amyloid formation in the Islandic form of hereditary cerebral hemorrhage with amyloidosis caused by an inherited mutation (L68Q) within the coding region of CST3. The production rate of cystatin C is remarkably constant and its plasma concentration can therefore be used as a reliable measure of the glomerular filtration rate (Grubb, Clinical Nephrology, Vol. 38, Suppl. No.1, 20 - 27, 1992).

The role of amyloid proteins, in particular amyloid  $\beta$  protein, in neurodegenerative disorders has been extensively described in literature (see e.g. Harper J.D. and Lansbury P.T., Annu. Rev. Biochem., 66, 385 - 407, 1997). The family of A $\beta$  variants is derived from the amyloid precursor protein (APP), a ubiquitous ca. 700-amino acid cell-surface protein. Two variants, A $\beta$ 1-40 and A $\beta$ 1-42, which differ by truncation at the carboxyl terminus are the predominant amyloid plaque proteins.

It might be preferred that said subject has previously been determined to have one or more factors indicating that such subject is afflicted with AMD.

In a further preferred embodiment, A $\beta$ 1-40 and/or A $\beta$ 1-42 are determined as amyloid protein.

In preferred embodiments, the sample is taken from the eye of said subject. It is particularly preferred to take a sample from material located between the plasma membrane and basal lamina of the retinal pigment epithelium and/or from material located between the basal lamina of the retinal pigment epithelium and the inner collagenous zone of Bruch's membrane.



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According to the present invention, an increase of the level of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and/or a transcription product of a gene coding for said amyloid protein in said sample from the subject relative to a reference value indicates a diagnosis, or prognosis, or increased risk of said age-related macular degeneration in said subject. As shown in Example 1, a significantly higher frequency of the CST3 BB genotype is observed in patients with exudative AMD. A specific feature associated with this genotype might be an abnormal level of the active form of Cystatin C in specific tissues and bodily fluids.

In preferred embodiments, measurement of the level of transcription products of the cystatin C gene or a gene coding for said amyloid protein is performed using Northern blots with probes specific for said genes. Quantitative PCR with primer combinations to amplify gene-specific sequences from cDNA obtained by reverse transcription of RNA extracted from said sample of a subject can also be applied. These techniques are known to those of ordinary skill in the art (see e.g. Watson et al., Rekombinierte DNA, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg, 1993; Watson et al., Recombinant DNA, 2nd ed., W. H. Freeman and Company, 1992).

In preferred embodiments, said level or activity of cystatin C or said amyloid protein is detected using an immunoassay. These assays can e.g. measure the amount of binding between cystatin C and an anti-cystatin C antibody by the use of enzymatic, chromodynamic, radioactive, or luminescent labels which are attached to either the anti-cystatin C antibody or a secondary antibody which binds the anti-cystatin C antibody. In addition, other high affinity ligands including cathepsin derivatives may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow et al., Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

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The antibody or ligand to be used should preferably specifically detect cystatin C or said amyloid protein. It is preferred that it does not substantially interact with any other protein present in said sample. It is particularly preferred to include specific antibodies or ligands which differentiate monomers from dimers or oligomers of cystatin C. The detection of the monomeric form of cystatin C may be more specific to age-related macular degeneration than measuring dimers or oligomers. Measures might be significantly improved with monomer-specific ELISAs.

Monoclonal antibodies capable of recognizing cystatin C or said amyloid proteins can be prepared using methods known in the art (see e.g. Köhler and Milstein, Nature 256, 495 - 497 1975; Kozbor et al., Immunol. Today 4, 72, 1983; Cole et al., Monoclonal antibodies and cancer therapy, Alan R. Liss, Inc., pp 77 - 98, 1985; Marks et al., J. Biol. Chem., 16007 - 16010, 1992; the contents of which are incorporated herein by reference). Such monoclonal antibodies or fragments thereof can also be produced by alternative methods known to those of skill in the art of recombinant DNA technology (see e.g. Sastry et al, PNAS 86: 5728, 1989; ; Watson et al., Rekombinierte DNA, 2nd ed., Spektrum Akademischer Verlag GmbH, 1993; Watson et al, Recombinant DNA, 2nd ed., W. H. Freeman and Company, 1992; the contents of which are incorporated herein by reference). Monoclonal antibodies useful in the methods of the invention are directed to an epitope of cystatin C or said amyloid protein, such that the complex formed between the antibody and cystatin C, or between the antibody and said amyloid protein, can be recognized in detection assays. The term "antibodies" encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, single chain antibodies as well as fragments thereof which specifically bind to cystatin C, or to an amyloid protein. It is particularly preferred to use specific antibodies that selectively detect cystatin C monomers, dimers or oligomers, respectively.

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High-affinity ligands can be prepared by using derivatives of cathepsins which are the natural substrates of cystatin C biological activity.

It is further preferred to determine e.g. the level of cystatin C and its activity on basis of an enzymatic assay. As described above, cystatin C is a cysteine protease inhibitor which binds to and regulates proteolytic activities of cathepsins. A suitable enzymatic assay can therefore be built upon the enzymatic activity of cathepsins indicating the absence or presence of different levels of cystatin C in its active, monomeric form. It is preferred to use amyloid precursor protein (APP) as a substrate. The generation of A-beta peptides as educts can be measured.

The determination of the level or activity of cystatin C, or of an amyloid protein, can also be performed on basis of a binding assay. Suitable binding partners include cathepsins or fragments thereof, peptides, peptidomimetics, antibodies and other chemical probes which can specifically recognize cystatin C or an amyloid protein. It is in general particularly preferred to determine cystatin C in its monomeric form. Suitable binding partners for an amyloid protein assay include e.g. peptides, peptidomimetics, antibodies and other chemical probes.

If luminescent labels are used in any detection assay, it is preferred to use a confocal optical set-up.

In preferred embodiments, the reference value can be that of a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for said amyloid protein in a sample from a subject not suffering from age-related macular degeneration. The healthy subject can be of the same weight, age, and gender as the subject who is being diagnosed or prognosed

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for AMD, or for whom an increased risk of becoming diseased with AMD is determined. In some cases, it might be preferred to use a reference value from the subject which is diagnosed.

In preferred embodiments, the subject can be a human, an experimental animal, e.g. a rat or a mouse, a domestic animal, or a non-human primate, e.g. a monkey. The experimental animal can be an animal model for a disorder, e.g. a transgenic mouse with an AMD pathology.

In another aspect, the invention features, a method of monitoring the progression of age-related macular degeneration in a subject. The method includes: determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for said amyloid protein in a sample from the subject; and comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status, thereby monitoring the progression of said age-related macular degeneration in said subject. In a preferred embodiment, the level, or the activity, or both said level and said activity, of at least one of said substances in a sample is determined at least twice, e.g. at two points which are weeks or months apart. The levels or activities at these two time points are compared in order to monitor the progression of AMD. It might further be preferred to compare a level, or an activity, or both said level and said activity of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein and a transcription product of a gene coding for said amyloid protein in said sample with a level, an activity, or both said level and said activity, of at least one of said substances in a series of samples taken from said subject over a period of time. In further preferred

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embodiments, said subject receives a treatment prior to one or more of said sample gatherings.

It might be preferred that said subject has previously been determined to have one or more factors indicating that such subject is afflicted with age-related macular degeneration.

In a further preferred embodiment, A $\beta$ 1-40 and/or A $\beta$ 1-42 are determined as amyloid proteins.

In preferred embodiments, the sample is taken from the eye of said subject. It is particularly preferred to take a sample from material located between the plasma membrane and basal lamina of the retinal pigment epithelium and/or from material located between the basal lamina of the retinal pigment epithelium and the inner collagenous zone of Bruch's membrane.

According to the present invention, an increase of the level of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and/or a transcription product of a gene coding for said amyloid protein in said sample from the subject relative to a reference value indicates a diagnosis, or prognosis, or increased risk of said AMD in said subject. As shown in Example 1, a significantly higher frequency of the CST3 BB genotype is observed in patients with exudative AMD. A specific feature associated with this genotype might be an abnormal level of the active form of Cystatin C in specific tissues and bodily fluids.

In preferred embodiments, measurement of the level of transcription products of the cystatin C gene and/or said gene coding for amyloid proteins is performed using Northern blots with probes specific for said genes. Quantitative PCR with primer combinations to amplify gene-specific sequences from cDNA obtained by reverse transcription of RNA extracted from the subject can also be

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applied. These techniques are known to those of ordinary skill in the art (see e.g. Watson et al., *Rekombinierte DNA*, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg, 1993; Watson et al., *Recombinant DNA*, 2nd ed., W. H. Freeman and Company, 1992).

In preferred embodiments, said level or activity of cystatin C and/or an amyloid protein is detected using an immunoassay. These assays can measure e.g. the amount of binding between cystatin C and an anti-cystatin C antibody by the use of enzymatic, chromodynamic, radioactive, or luminescent labels which are attached to either the anti-cystatin C antibody or a secondary antibody which binds the anti-cystatin C antibody. In addition, other high affinity ligands including cathepsin derivatives may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

The antibody or ligand to be used should preferably specifically detect cystatin C or said amyloid proteins. It is preferred that it does not substantially interact with any other protein present in said sample. It is particularly preferred to include specific antibodies or ligands which differentiate monomers from dimers or oligomers of cystatin C. The detection of the monomeric form of cystatin C may be more specific to AMD than measuring dimers or oligomers. Measures might be significantly improved with monomer-specific ELISAs.

Monoclonal antibodies capable of recognizing cystatin C or said amyloid proteins can be prepared using methods known in the art (see e.g. Köhler and Milstein, *Nature* 258, 495 - 497 1975; Kozbor et al., *Immunol. Today* 4, 72, 1983; Cole et al., *Monoclonal antibodies and cancer therapy*, Alan R. Liss, Inc., pp 77 - 98, 1985; Marks et al., *J. Biol. Chem.*, 16007 - 16010, 1992; the contents of which are incorporated herein by reference). Such monoclonal

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antibodies or fragments thereof can also be produced by alternative methods known to those of skill in the art of recombinant DNA technology (see e.g. Sastry et al, PNAS 86: 5728, 1989; Watson et al., Rekombinierte DNA, 2nd ed., Spektrum Akademischer Verlag GmbH, 1993; Watson et al., Recombinant DNA, 2nd ed., W. H. Freeman and Company, 1992; the contents of which are incorporated herein by reference). Monoclonal antibodies useful in the methods of the invention are directed to an epitope of cystatin C or said amyloid proteins such that the complex formed between the antibody and cystatin C, or between the antibody and said amyloid protein, can be recognized in detection assays. The term "antibodies" encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, single chain antibodies as well as fragments thereof which specifically bind to cystatin C, or to said amyloid protein. It is particularly preferred to use specific antibodies that selectively detect cystatin C monomers, dimers or oligomers, respectively. High-affinity ligands can be prepared by using derivatives of cathepsins which are the natural substrates of cystatin C biological activity.

It is further preferred to determine e.g. the level of cystatin C and its activity on basis of an enzymatic assay. As described above, cystatin C is a cysteine protease inhibitor which binds to and regulates proteolytic activities of cathepsins. A suitable enzymatic assay can therefore be built upon the enzymatic activity of cathepsins indicating the absence or presence of different levels of cystatin C in its active, monomeric form. It is preferred to use amyloid precursor protein (APP) as a substrate. The generation of A-beta peptides as products can be measured.

The determination of the level or activity of cystatin C or said amyloid proteins can also be performed on basis of a binding assay. Suitable binding partners include cathepsins or fragments thereof, peptides, peptidomimetics, antibodies and other chemical probes which can specifically recognize cystatin C. It is in

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general particularly preferred to determine cystatin C in its monomeric form. Suitable binding partners for an amyloid protein assay include e.g. peptides, peptidomimetics, antibodies and other chemical probes.

If luminescent labels are used in any detection assay, it is preferred to use a confocal optical set-up.

In preferred embodiments, the reference value can be that of a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in a sample, preferably an eye, from a subject not suffering from age-related macular degeneration. The healthy subject can be of the same weight, age, and gender as the subject who is being diagnosed or prognosed for AMD, or for whom an increased risk of becoming diseased with AMD is determined. In some cases, it might be preferred to use a reference value from the subject which is monitored.

In preferred embodiments, the subject can be a human, an experimental animal, e.g. a rat or a mouse, a domestic animal, or a non-human primate, e.g. a monkey. The experimental animal can be an animal model for a disorder, e.g. a transgenic mouse with an AMD pathology.

In another aspect, the invention features, a method of evaluating a treatment for age-related macular degeneration. The method includes: determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from a group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for an amyloid protein in a sample obtained from said subject being treated for said AMD; and comparing said



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level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status, thereby evaluating said treatment for said AMD. It is further preferred that a level, or an activity, or both said level and said activity of at least one of said substances is determined, before and after said treatment is administered to said subject.

In a further preferred embodiment, A $\beta$ 1-40 and/or A $\beta$ 1-42 are determined as amyloid protein.

In preferred embodiments, the sample is taken from the eye of a subject. It is particularly preferred to take a sample from material located between the plasma membrane and basal lamina of the retinal pigment epithelium and/or from material located between the basal lamina of the retinal pigment epithelium and the inner collagenous zone of Bruch's membrane.

It might be preferred that said subject has previously been determined to have one or more factors indicating that such subject is afflicted with AMD.

According to the present invention, an increase of the level of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and/or a transcription product of a gene coding for said amyloid protein in said sample from the subject relative to a reference value indicates a diagnosis, or prognosis, or increased risk of said AMD in said subject. As shown in Example 1, a significantly higher frequency of the CST3 BB genotype is observed in patients with exudative AMD. A specific feature associated with this genotype might be an abnormal level of the active form of Cystatin C in specific tissues and bodily fluids.

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In preferred embodiments, measurement of the level of transcription products of the cystatin C gene, or of said gene coding for an amyloid protein, is performed using Northern blots with probes specific for said genes. Quantitative PCR with primer combinations to amplify gene-specific sequences from cDNA obtained by reverse transcription of RNA extracted from a subject can also be applied. These techniques are known to those of ordinary skill in the art (see e.g. Watson et al., *Rekombinierte DNA*, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg, 1993; Watson et al., *Recombinant DNA*, 2nd ed., W. H. Freeman and Company, 1992).

In preferred embodiments, said level or activity of cystatin C, or of an amyloid protein, is detected using an immunoassay. These assays can e.g. measure the amount of binding between cystatin C and an anti-cystatin C antibody by the use of enzymatic, chromodynamic, radioactive, or luminescent labels which are attached to either the anti-cystatin C antibody or a secondary antibody which binds the anti-cystatin C antibody. In addition, other high affinity ligands including cathepsin derivatives may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

The antibody or ligand to be used should preferably specifically detect cystatin C or an amyloid protein. It is preferred that it does not substantially interact with any other protein present in said sample. It is particularly preferred to include specific antibodies or ligands which differentiate monomers from dimers or oligomers of cystatin C. The detection of the monomeric form of cystatin C may be more specific to AD than measuring dimers or oligomers. Measures might be significantly improved with monomer-specific ELISAs.

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Monoclonal antibodies capable of recognizing cystatin C or an amyloid protein can be prepared using methods known in the art (see e.g. Köhler and Milstein, *Nature* 256, 495 - 497 1975; Kozbor et al., *Immunol. Today* 4, 72, 1983; Cole et al., *Monoclonal antibodies and cancer therapy*, Alan R. Liss, Inc., pp 77 - 96, 1985; Marks et al., *J. Biol. Chem.*, 16007 - 16010, 1992; the contents of which are incorporated herein by reference). Such monoclonal antibodies or fragments thereof can also be produced by alternative methods known to those of skill in the art of recombinant DNA technology (see e.g. Sastry et al, *PNAS* 86: 5728, 1989; Watson et al., *Rekombinierte DNA*, 2nd ed., Spektrum Akademischer Verlag GmbH, 1993; Watson et al., *Recombinant DNA*, 2nd ed., W. H. Freeman and Company, 1992; the contents of which are incorporated herein by reference). Monoclonal antibodies useful in the methods of the invention are directed to an epitope of cystatin C or of an amyloid protein, such that the complex formed between the antibody and cystatin C, or between the antibody and said amyloid protein, can be recognized in detection assays. The term "antibodies" encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, single chain antibodies as well as fragments thereof which specifically bind to cystatin C, or to an amyloid protein. It is particularly preferred to use specific antibodies that selectively detect cystatin C monomers, dimers or oligomers, respectively. High-affinity ligands can be prepared by using derivatives of cathepsins which are the natural substrates of cystatin C biological activity.

It is further preferred to determine e.g. the level of cystatin C and its activity on basis of an enzymatic assay. As described above, cystatin C is a cysteine protease inhibitor which binds to and regulates proteolytic activities of cathepsins. A suitable enzymatic assay can therefore be built upon the enzymatic activity of cathepsins indicating the absence or presence of different levels of cystatin C in its active, monomeric form. It is preferred to use amyloid

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precursor protein (APP) as a substrate. The generation of A-beta peptides as educts can be measured.

The determination of the level or activity of cystatin C, or an amyloid protein, can also be performed on basis of a binding assay. Suitable binding partners include cathepsins or fragments thereof, peptides, peptidomimetics, antibodies and other chemical probes which can specifically recognize cystatin C. It is in general particularly preferred to determine cystatin C in its monomeric form. Suitable binding partners for amyloid protein include e.g. peptides, peptidomimetics, antibodies and other chemical probes.

If luminescent labels are used in any detection assay, it is preferred to use a confocal optical set-up.

In preferred embodiments, the reference value can be that of a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for said amyloid protein in a sample, preferably an eye, from a subject not suffering from age-related macular degeneration. The healthy subject can be of the same weight, age, and gender as the subject who is being diagnosed or prognosed for AMD, or for whom an increased risk of becoming diseased with AMD is determined. In some cases, it might be preferred to use a reference value from the subject which is under treatment.

In preferred embodiments, the subject can be a human, an experimental animal, e.g. a rat or a mouse, a domestic animal, or a non-human primate, e.g. a monkey. The experimental animal can be an animal model for a disorder, e.g. a transgenic mouse with an AMD pathology.

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In another aspect, the invention features, a method of diagnosing or prognosing age-related macular degeneration in a subject, or determining whether a subject is at increased risk of becoming diseased with age-related macular degeneration. The method includes: determining the presence or absence of a mutation or polymorphism in a cystatin C gene or its non-coding regulatory elements in a sample from said subject, thereby diagnosing, or prognosing, or determining an increased risk of AMD in said subject. Such a mutation can e.g. be a substitution, deletion or addition of at least one base. Such mutations may result in "mis-sense" information or in "non-sense" information associated with a termination codon or a frame shift. Polymorphisms and allele variations occur more frequently in the general population but induce in principle the identical genetic alterations. Such mutations or polymorphisms may be found within the promoter region, an example of which is the Sst II polymorphic site in the promoter region of the human cystatin C gene (CST 3). It is preferred to determine the presence of a B allele in the cystatin C gene. The human cystatin C gene, called CST3, has been sequenced and its A and B alleles were also described (Baibin et al., Biol. Chem. Hoppe-Seyler, Vol. 373, 471 - 476, 1992; Abrahamson et al., Biochem. J. 268, 287 - 294, 1990; Abrahamson et al., Hum. Genet. 82, 223 - 228, 1989; the contents of these publications are incorporated herein by reference). The presence of at least one B allele in said cystatin C gene or its non-coding regulatory elements, particularly in its promoter, indicates said subject and potentially its descendants are at increased risk of developing age-related macular degeneration. In particular, homozygous CST 3 B/B subjects are at increased risk of developing AMD. The data shown in Example 1 indicate an association of CST3 B/B genotype with AMD.

Contradictory results were reported for a possible role of ApoE polymorphism in AMD. Some authors have reported a lower frequency of the  $\epsilon 4$  allele in subgroups of AMD while other reports could not confirm this association. The

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$\epsilon 2$  allele was reportedly more frequent in AMD patients. Allelic variations in the ABCR gene were proposed to be associated with advanced atrophic AMD. However, other authors have found no evidence to support this hypothesis.

Determining the presence or absence of a mutation or polymorphism in a cystatin C gene in a sample from said subject may comprise determining a partial nucleotide sequence of the DNA from said subject, said partial nucleotide sequence indicating the presence or absence of said mutation or polymorphism. It may further be preferred to perform a polymerase chain reaction with the DNA from said subject and subsequent restriction analysis to determine the presence or absence of said mutation or polymorphism. In a further preferred embodiment, primers depicted in SEQ ID NO. 1 and SEQ ID NO. 2 are used for amplifying the promoter region of the human cystatin C gene in order to subsequently analyze the Sst II polymorphic sites herein.

In another preferred embodiment, the method further includes: determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for said amyloid protein in a sample from said subject; and comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value.

For the determination of mutations or polymorphisms in the cystatin C gene, it is preferred to use DNA from body cells, in particular white blood cells.

In another aspect, the invention features, a kit for diagnosis, or prognosis, or determination of increased risk of age-related macular degeneration in a subject.

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**Said kit comprises:**

- (a) at least one reagent which is selected from the group consisting of reagents that selectively detect cystatin C, reagents that selectively detect transcription products of a cystatin C gene, reagents that selectively detect an amyloid protein, reagents that selectively detect transcription products of a gene coding for an amyloid protein, and reagents that selectively detect mutations or polymorphisms in the cystatin C gene or its non-coding regulatory elements; and
- (b) instructions for diagnosing, or prognosing, or determining whether said subject is at increased risk of developing, age-related macular degeneration by
  - (i) detecting a level, or an activity, or both said level and said activity, of said cystatin C, or of said transcription products of said cystatin C gene, or of said amyloid protein, or of said transcription products of a gene coding for an amyloid protein, in a sample from said subject; or detecting a presence or absence of mutations or polymorphisms in said cystatin C gene or its non-coding regulatory elements in a sample from said subject; and
  - (ii) diagnosing or prognosing, or determining whether said subject is at increased risk of developing, age-related macular degeneration, wherein
    - a varied level, or activity, or both said level and said activity, of said cystatin C, or of said transcription products of said cystatin C gene, or of an amyloid protein, or of a transcription product of a gene coding for an amyloid protein, compared to a reference value representing a known health status;
    - or a level, or activity, or both said level and said activity,

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of said cystatin C, or of said transcription products of said cystatin C gene, or of said amyloid protein, or of said transcription products of a gene coding for an amyloid protein similar or equal to a reference value representing a known disease status;  
or the presence of a mutation or polymorphism in said cystatin C gene or its non-coding regulatory elements indicates a diagnosis, or prognosis, or an increased risk of age-related macular degeneration.

It is preferred that said at least one reagent and said instructions are packaged in a single container. In preferred embodiments, said reference value is that of a level, or an activity, or both said level and said activity, of at least one substance which is selected from the above mentioned group in a sample from a subject not suffering from said AMD. The healthy subject can be of the same weight, age, and gender as the subject who is being diagnosed, or prognosed for AMD, or for whom an increased risk of developing AMD is determined. In some cases, it might be preferred to use a reference value of the subject which is to be diagnosed. Said kit suitable for commercial manufacture and sale can still further include appropriate standards, positive and negative controls.

In further preferred embodiments, the presence of at least one B allele in said cystatin C gene, in particular the presence of the B/B genotype, indicates a diagnosis, or prognosis, or an increased risk of age-related macular degeneration. In order to exclude a false positive diagnosis, it should be remarked that a mutation or polymorphism in the cystatin C gene in the codon for leucine at position 68 which abolishes an A/*u*l restriction site is not indicative for age-related macular degeneration, but for hereditary cystatin C amyloid angiopathy.



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Determining the presence or absence of a mutation or polymorphism in a cystatin C gene or in its non-coding regulatory elements in a sample from said subject may comprise determining a partial nucleotide sequence of the DNA from said subject, said partial nucleotide sequence indicating the presence or absence of said mutation or polymorphism. It may further be preferred to perform a polymerase chain reaction with the DNA from said subject and subsequent restriction analysis to determine the presence or absence of said mutation. In a further preferred embodiment, primers depicted in SEQ ID NO. 1 and SEQ ID NO. 2 are used for amplifying the promoter region of the human cystatin C gene in order to subsequently analyze the Sst II polymorphic sites herein.

In preferred embodiments, measurement of the level of transcription products of the cystatin C gene, or of a gene coding for an amyloid protein, is performed using Northern blots with probes specific for said genes. Quantitative PCR with primer combinations to amplify gene-specific sequences from cDNA obtained by reverse transcription of RNA extracted from body cells of a subject can also be applied. These techniques are known to those of ordinary skill in the art (see e.g. Watson et al., Rekombinierte DNA, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg, 1993; Watson et al., Recombinant DNA, 2nd ed., W. H. Freeman and Company, 1992).

For the determination of mutations or polymorphisms in the cystatin C gene, it is preferred to use DNA from body cells including fibroblasts and white blood cells. For the other analyses, it is particularly preferred to use samples taken from the eye, as explained above.

In preferred embodiments, said level or activity of said above mentioned substances is detected using an immunoassay. These assays can e.g. measure the amount of binding between cystatin C and an anti-cystatin C

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antibody by the use of enzymatic, chromodynamic, radioactive, or luminescent labels which are attached to either the anti-cystatin C antibody or a secondary antibody which binds the anti-cystatin C antibody. In addition, other high affinity ligands including cathepsin derivatives may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

The antibody or ligand to be used should preferably specifically detect cystatin C, or an amyloid protein. It is preferred that it does not substantially interact with any other protein present in said sample. It is particularly preferred to include specific antibodies or ligands which differentiate monomers from dimers or oligomers of cystatin C. The detection of the monomeric form of cystatin C may be more specific to AD than measuring dimers or oligomers. Measures might be significantly improved with monomer-specific ELISAs.

Monoclonal antibodies capable of recognizing cystatin C or an amyloid protein can be prepared using methods known in the art (see e.g. Köhler and Milstein, *Nature* 256, 495 - 497 1975; Kozbor et al., *Immunol. Today* 4, 72, 1983; Cole et al., *Monoclonal antibodies and cancer therapy*, Alan R. Liss, Inc., pp 77 - 96, 1985; Marks et al., *J. Biol. Chem.*, 16007 - 16010, 1992; the contents of which are incorporated herein by reference). Such monoclonal antibodies or fragments thereof can also be produced by alternative methods known to those of skill in the art of recombinant DNA technology (see e.g. Sastry et al, *PNAS* 86: 5728, 1989; Watson et al., *Rekombinierte DNA*, 2nd ed., Spektrum Akademischer Verlag GmbH, 1993; Watson et al., *Recombinant DNA*, 2nd ed., W. H. Freeman and Company, 1992; the contents of which are incorporated herein by reference). Monoclonal antibodies useful in the methods of the invention are directed to an epitope of cystatin C or an amyloid protein, such that the complex formed between the antibody and cystatin C, or between the

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antibody and said amyloid protein, can be recognized in detection assays. The term "antibodies" encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, single chain antibodies as well as fragments thereof which specifically bind to cystatin C, or to an amyloid protein. It is particularly preferred to use specific antibodies that selectively detect cystatin C monomers, dimers or oligomers, respectively. High-affinity ligands can be prepared by using derivatives of cathepsins which are the natural substrates of cystatin C biological activity.

It is further preferred to determine the level of cystatin C and its activity on basis of an enzymatic assay. As described above, cystatin C is a cysteine protease inhibitor which binds to and regulates proteolytic activities of cathepsins. A suitable enzymatic assay can therefore be built upon the enzymatic activity of cathepsins indicating the absence or presence of different levels of cystatin C in its active, monomeric form. It is preferred to use amyloid precursor protein (APP) as a substrate. The generation of A-beta peptides as educts can be measured.

The determination of the level or activity of cystatin C, or an amyloid protein, can also be performed on basis of a binding assay. Suitable binding partners include cathepsins or fragments thereof, peptides, peptidomimetics, antibodies and other chemical probes which can specifically recognize cystatin C. It is in general particularly preferred to determine cystatin C in its monomeric form. Suitable binding partners for an amyloid protein assay include e.g. peptides, peptidomimetics, antibodies and other chemical probes.

If luminescent labels are used in any detection assay, it is preferred to use a confocal optical set-up.

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In preferred embodiments, the subject can be a human, an experimental animal, e.g. a rat or a mouse, a domestic animal, or a non-human primate, e.g. a monkey. The experimental animal can be an animal model for a disorder, e.g. a transgenic mouse with an AMD pathology.

In preferred embodiments, said kit can also be used in monitoring a progression of AMD in a subject or in monitoring success or failure of a therapeutic treatment of said subject.

In another aspect, the invention features a method of treating or preventing AMD in a subject comprising administering to said subject in a therapeutically effective amount an agent or agents which directly or indirectly affect a biological activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein.

It is preferred that said agent or agents reduce a biological activity, or level, or both said activity and level, of at least one of the mentioned substances.

In preferred embodiments, said agents bind or inhibit cystatin C, as e.g. cathepsin derivatives or cystatin C analogs. In further preferred embodiments, said agents inhibit the formation of amyloid plaques in said subject's eyes.

In preferred embodiments, the method comprises the application of per se known methods of gene therapy and/or antisense nucleic acid technology to administer said agent or agents.

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In general, gene therapy includes several approaches: molecular replacement of a mutated gene, addition of a new gene resulting in the synthesis of a therapeutic protein, and modulation of endogeneous cellular gene expression by drugs. Gene-transfer techniques are described in detail (see e.g. Behr, Acc. Chem. Res. 26, 274 - 278, 1993; Mulligan, Science 260, 926 - 931, 1993; the contents of which are incorporated herein by reference) and include direct gene-transfer techniques such as mechanical microinjection of DNA into a cell as well as indirect techniques employing biological vectors (like recombinant viruses, especially retroviruses) or model liposomes, or techniques based on transfection with DNA coprecipitation with polycations, cell membrane perturbation by chemical (solvents, detergents, polymers, enzymes) or physical means (mechanic, osmotic, thermic, electric shocks).

In particular, the invention features a method of treating or preventing age-related macular degeneration by means of antisense nucleic acid therapy, i.e. the down-regulation of an inappropriately expressed or defective gene by the introduction of antisense nucleic acids or derivatives thereof into certain critical cells (see e.g. Gillespie, DN & P 5(7), 389 - 395, 1992; Agrawal, Tibtech 13, 197 - 199, 1995; Crooke, Bio/Technology 10, 882 - 886, 1992; the contents of which are incorporated herein by reference). Apart from hybridization strategies, the application of ribozymes, i.e. RNA molecules that act as enzymes, destroying RNA that carries the message of disease has also been described (see e.g. Barinaga, Science, 262, 1512 - 1514, 1993; the contents of which are incorporated herein by reference). In preferred embodiments, the subject to be treated is a human and therapeutic antisense nucleic acids or derivatives thereof are directed against the human cystatin C gene CST-3, its non-coding regulatory elements, or transcription products of CST-3. Cell penetration can be performed by known strategies such as coupling of antisense nucleic acids and derivatives thereof to carrier particles, or the above described techniques. Strategies for administering targeted therapeutic oligodeoxynucleotides are known to those of skill in the art (see e.g. Wickstrom,

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Tibtech, 10, 281 - 287, 1992; the contents of which are incorporated herein by reference). In some cases, delivery can be performed by mere topical application. Further approaches are directed to intracellular expression of antisense RNA. In this strategy, cells are transformed *ex vivo* with a recombinant gene that directs the synthesis of an RNA that is complementary to a region of the target nucleic acid. Therapeutic use of intracellularly expressed antisense RNA is procedurally similar to gene therapy.

In preferred embodiments, the method comprises grafting donor cells into the eye of said subject, said subject or donor cells preferably treated so as to minimize or reduce graft rejection, wherein said donor cells are genetically modified by insertion of at least one transgene encoding said agent or agents. Said transgene might be carried by a viral vector, in particular a retroviral vector. The transgene can be inserted into the donor cells by a nonviral physical transfection of DNA encoding a transgene, in particular by microinjection. Insertion of the transgene can also be performed by electroporation, chemically mediated transfection, in particular calcium phosphate transfection, liposomal mediated transfection, etc.

In preferred embodiments, said agent is a therapeutic protein which can be administered to said subject, preferably a human, by a process comprising introducing subject cells into said subject, said subject cells having been treated *in vitro* to insert a DNA segment encoding said therapeutic protein, said subject cells expressing *in vivo* in said subject a therapeutically effective amount of said therapeutic protein. Said DNA segment can be inserted into said cells *in vitro* by a viral vector, in particular a retroviral vector.

In preferred embodiments, the therapeutic nucleic acid or protein reduces amyloid formation by interacting with a cystatin C gene, its transcription products, or cystatin C. It is preferred that said amyloid is  $\beta$ -amyloid derived by

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proteolytic processing of the amyloid precursor protein (APP) known in Alzheimer's disease.

In preferred embodiments, the subject can be a human, an experimental animal, e.g. a rat or a mouse, a domestic animal, or a non-human primate, e.g. a monkey. The experimental animal can be an animal model for a disorder, e.g. a transgenic mouse with an AMD pathology.

In another aspect, the invention features an agent which directly or indirectly affects a biological activity, or level, or both said activity and level, of at least one of the above mentioned substances.

It is preferred that said agent or agents reduce(s) a biological activity, or level, or both said activity and level, of at least one substance which is selected from the above mentioned substances. In preferred embodiments, the agent is a therapeutic nucleic acid or protein which reduces amyloid formation by interacting with a cystatin C gene, its transcription products, or cystatin C. It is preferred that said amyloid is  $\beta$ -amyloid derived by proteolytic processing of the amyloid precursor protein (APP) known in Alzheimer's disease.

In a further aspect, the invention features a medicament comprising an agent which directly or indirectly affects a biological activity, or level, or both said activity and level, of at least one substance which is mentioned above. It is preferred that said agent reduces a biological activity, or level, or both said activity and level, of at least one of said substances.

In another aspect, the invention features an agent which directly or indirectly affects a biological activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, a transcription product of a cystatin C gene, cystatin C, a gene coding for an

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amyloid protein, a transcription product of a gene coding for an amyloid protein, and an amyloid protein, for treating or preventing age-related macular degeneration. It is preferred that said agent or agents reduce a biological activity, or level, or both said activity and level, of at least one of said substances. In preferred embodiments, the agent is a therapeutic nucleic acid or protein which reduces amyloid formation by interacting with a cystatin C gene, its transcription products, or cystatin C. It is preferred that said amyloid is  $\beta$ -amyloid derived by proteolytic processing of the amyloid precursor protein (APP) known in Alzheimer's disease.

In a further aspect, the invention features the use of an agent which directly or indirectly affects a biological activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, a transcription product of a gene coding for an amyloid protein, and an amyloid protein, for a preparation of a medicament for treating or preventing age-related macular degeneration. It is preferred that said agent or agents reduce(s) a biological activity, or level, or both said activity and level, of at least one of said substances. In preferred embodiments, the agent is a therapeutic nucleic acid or protein which reduces amyloid formation by interacting with a cystatin C gene, its non-coding regulatory elements, its transcription products, or cystatin C. It is preferred that said amyloid is  $\beta$ -amyloid derived by proteolytic processing of the amyloid precursor protein (APP) known in Alzheimer's disease.

In another aspect, the invention features a method for identifying an agent that directly or indirectly affects an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding



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regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein comprising the steps of:

- (a) providing a sample containing at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein;
- (b) contacting said sample with at least one agent;
- (c) comparing an activity, or level, or both said activity and level, of at least one of said substances before and after said contacting.

It is preferred that said agent reduces an activity, or level, or both said activity and level, of at least one of said substances.

In preferred embodiments, measurement of the level of transcription products of a cystatin C gene, or of a gene coding for an amyloid protein, is performed using Northern blots with probes specific for said genes. Quantitative PCR with primer combinations to amplify gene-specific sequences from cDNA obtained by reverse transcription of RNA extracted from a subject can also be applied. These techniques are known to those of ordinary skill in the art (see e.g. Watson et al., Rekombinierte DNA, 2nd edition, Spektrum Akademischer Verlag GmbH, Heidelberg, 1993; Watson et al., Recombinant DNA, 2nd ed., W. H. Freeman and Company, 1992).

In preferred embodiments, said level or activity of cystatin C, or of an amyloid protein, is detected using an immunoassay. These assays can e.g. measure the amount of binding between cystatin C and an anti-cystatin C antibody by the use of enzymatic, chromodynamic, radioactive, or luminescent labels which are attached to either the anti-cystatin C antibody or a secondary antibody

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which binds the anti-cystatin C antibody. In addition, other high affinity ligands including cathepsin derivatives may be used. Immunoassays which can be used include e.g. ELISAs, Western blots and other techniques known to those of ordinary skill in the art (see Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

The antibody or ligand to be used should preferably specifically detect cystatin C or an amyloid protein. It is preferred that it does not substantially interact with any other protein present in said sample. It is particularly preferred to include specific antibodies or ligands which differentiate monomers from dimers or oligomers of Cystatin C.

Monoclonal antibodies capable of recognizing cystatin C, or an amyloid protein, can be prepared using methods known in the art (see e.g. Köhler and Milstein, *Nature* 258, 495 - 497 1975; Kozbor et al., *Immunol. Today* 4, 72, 1983; Cole et al., *Monoclonal antibodies and cancer therapy*, Alan R. Liss, Inc., pp 77 - 96, 1985; Marks et al., *J. Biol. Chem.*, 16007 - 16010, 1992; the contents of which are incorporated herein by reference). Such monoclonal antibodies or fragments thereof can also be produced by alternative methods known to those of skill in the art of recombinant DNA technology (see e.g. Sastry et al, *PNAS* 86: 5728, 1989; Watson et al., *Rekombinierte DNA*, 2nd ed., Spektrum Akademischer Verlag GmbH, 1993; Watson et al., *Recombinant DNA*, 2nd., W. H. Freeman and Company, 1992; the contents of which are incorporated herein by reference). Monoclonal antibodies useful in the methods of the invention are directed to an epitope of cystatin C or an amyloid protein, such that the complex formed between the antibody and cystatin C, or between the antibody and said amyloid protein, can be recognized in detection assays. The term "antibodies" encompasses all forms of antibodies known in the art, such as polyclonal, monoclonal, chimeric, recombinatorial, single chain antibodies as well as fragments thereof which specifically bind to cystatin C or said amyloid

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protein. It is particularly preferred to use specific antibodies that selectively detect cystatin C monomers, dimers or oligomers, respectively. High-affinity ligands can be prepared by using derivatives of cathepsins which are the natural substrates of cystatin C biological activity.

It is further preferred to determine the level of cystatin C and its activity on basis of an enzymatic assay. As described above, cystatin C is a cysteine protease inhibitor which binds to and regulates proteolytic activities of cathepsins. A suitable enzymatic assay can therefore be built upon the enzymatic activity of cathepsins indicating the absence or presence of different levels of cystatin C in its active, monomeric form. It is preferred to use amyloid precursor protein (APP) as a substrate. The generation of A-beta peptides, in particular A-beta 40 and A-beta 42 peptides, as educts can be measured.

The determination of the level or activity of cystatin C, or an amyloid protein, can also be performed on basis of a binding assay. Suitable binding partners include cathepsins or fragments thereof, peptides, peptidomimetics, antibodies and other chemical probes which can specifically recognize cystatin C, or a variant thereof. It is in general particularly preferred to determine cystatin C in its monomeric form. Suitable binding partners for an amyloid protein assay include e.g. peptides, peptidomimetics, antibodies and other chemical probes.

If luminescent labels are used in any detection assay, it is preferred to use a confocal optical set-up.

Other features and advantages of the invention will be apparent from the following detailed description of the examples, and from the claims.

Table 1 shows the number of Patients with AMD and control subjects.

Table 2 shows the *CST3* genotype distribution.

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Table 3 shows the *CST3* genotype distribution among males.

Table 4 shows the *CST3* genotype distribution among females.

Table 5 shows odds ratios for exudative AMD with *CST3* genotype BB dependent on gender.

Figure 1 depicts a schematic illustration of the human cystatin C gene with the exons numbered and shown as filled boxes as well as the three different alleles (A, B and C) with their respective nucleotide sequences given around the mutations in the promotor region. Nucleotide numbering for the base substitutions relates to the start site for cystatin C translation (+1 - +3 equals initiator methionine codon). The polymorphic *Sst* II and *Dde* I sites are underlined, and expected lengths of DNA fragments after respective cleavage are indicated.

Figure 2 depicts the possible roles of cystatin C and the *Aspergillus japonicus* cysteine proteinase inhibitor E-64 in the amyloid precursor protein (APP) processing and generation of the amyloid  $\beta$  peptide ( $A\beta$ ).

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### **Example 1**

#### **Patients and Methods**

167 patients with the clinical diagnosis of exudative AMD of all types, classic CNV, occult CNV and pigment epithelial detachment were selected. 268 unrelated age-matched control subjects were randomly selected to represent a sample of the general population, and therefore can be expected to develop AMD at the population rate (Table 1). The possibility of selecting elderly control subjects without signs of AMD was not chosen because this would require the differentiation of features associated with normal aging from early AMD. This distinction is difficult and its theoretical basis is weak. Informed consent was obtained from all patients and controls. Inclusion criteria for patients were: Diagnosis of uni- or bilateral neovascular AMD by using fluorescein angiography and absence of other retinal dystrophies or diseases that may be associated with the development of CNV.

A comprehensive ophthalmologic examination which included visual acuity measurement, fundus examination and fluorescein angiography was done in patients. Age at presentation and gender were recorded for both patients and control subjects. No age of onset of AMD was recorded for patients because due to the lingering course of the early forms often no definite date can be given.

Fluorescein angiographic photographs were evaluated according to the guidelines of the international classification and grading system of the International ARM epidemiological study group by two independent graders. Grading results were subsequently reviewed by an independent grader (The International ARM Epidemiological Study Group. An International classification and grading system for age-related maculopathy and age-related macular degeneration. Surv Ophthalmol. 1985; 39(5): 367-374). Inclusion criteria for the

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patient group were the presence of any form of neovascular maculopathy secondary to age-related macular degeneration including classic CNV, occult CNV, pigment epithelial detachment, or a combination of any of the above in one or both eyes. If only one eye was affected by neovascular AMD, the other eye had to present with phenomena of early AMD including more than 5 hard drusen, soft drusen, pigmentary abnormalities, or with advanced atrophic AMD (geographic atrophy) to ensure that AMD was the cause of neovascular maculopathy. In cases of disagreement between the two initial graders the conclusive grading was done by a the reviewing grader. To avoid bias, all graders were blinded with respect to the results of the genotype analyses.

Blood was collected from peripheral veins into EDTA tubes from patients and control subjects. Genomic DNA was extracted from isolated leukocytes using a standard salt precipitation technique and its concentration was determined spectrophotometrically. The PCR product was generated using the primers 024, TGGGAGGGACGAGGCGTTCC and 1206R, TCCATGGGGCCTCCCACCAG. A 10 µl polymerase chain reaction was performed, containing 0.4 µl of suspended genomic DNA, 500 nM of each forward and reverse primer, 1.5 mM magnesium chloride, 1 µl 10x buffer, 200 µM deoxyribonucleoside triphosphate, 0.4 U Taq polymerase (Gibco, Gaithersburg, MD), 0.5 µl of 5% dimethyl sulfoxide and 6.7 µl H<sub>2</sub>O. For a negative control, no DNA was added to the PCR reaction contents. The reactions were denaturated for 45 seconds at 95°C, then DNA was amplified for 13 cycles (15 seconds at 95°C, 30 seconds at 68°C reduced by 1°C per cycle and 30 seconds at 72°C) followed by 23 cycles (15 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C) then followed by a final 5-minute extension at 72°C. The PCR product is a 318 bp DNA fragment, 1 µl of each PCR product was electrophoresed on a 2.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination. After each PCR reaction, the remaining 9 µl of PCR product was used for enzyme digestion with

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10 units *Sac II* (MBI Fermentas, Vilnius, Lithuania) in 10 mM  $MgCl_2$ , 10 mM Tris-HCl, 0.2 mg bovine serum albumin (BSA) at pH 7.5 in 9  $\mu$ l purified water at 37°C overnight. Enzyme digestion revealed fragment sizes of 41, 51 and 226 bp from the A-allele and 127 and 191 bp from the B-allele, respectively. Haplotypes were confirmed by direct sequencing of PCR products from individuals with the genotypes AA, AB and BB. The digestion products were electrophoresed and visualized in the same manner as described for PCR products. They can easily be distinguished on electrophoresis.

Statistical analyses were done with SPSS (SPSS Inc., Chicago, IL). The non-parametric Pearson chi-square test was used to compare the *CST3*-BB bearers versus nonbearers consisting of the genotypes AA and AB ( $df=1$ ). Kaplan-Meier survival analysis was performed on data obtained from 167 patients and 268 controls. P-values less than 0.05 were considered as statistically significant. Means and standard deviations were calculated for age of patients and controls.

## Results

The analysis of demographic data revealed that age at presentation was similar for the male and female subgroup of AMD patients and control subjects (Table 1). Both genders were separately age-matched since the number of male and female subjects among patients and controls was unequally distributed ( $p<0.0001$ ). The genotype frequencies of *CST3* haplotypes were in Hardy-Weinberg equilibrium in both patients and controls.

Simultaneous genotyping of three polymorphic *SacII* restriction sites in the 5'-region of *CST3* covered by a single PCR fragment revealed a strong linkage disequilibrium between all three polymorphic *SacII* sites. The observed haplotypes were defined by either concomitant *SacII* restriction both 80 bp upstream of the transcription start sites and in the penultimate codon of the

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sequence that encodes the signal peptide (allele A), or by exclusive cleavage downstream of the transcription start sites (allele B).

The frequency of homozygous haplotype *CST3* BB genotype differed significantly between patients with exudative AMD and control subjects ( $p=0.0228$ ; Table 2). The frequency of this genotype was 0.066 for the entire patients group compared with 0.022 for control subjects overall. When subgroups for both genders were formed the frequency of the BB genotype was 0.113 in male patients and 0.024 in male controls. There was a statistically significant difference of the BB genotype distribution in the male subgroup ( $p=0.0119$ ; Fisher's exact test:  $p=0.0201$ ; Table 3). The frequency of the BB genotype in the female subgroup was 0.044 in patients versus 0.021 in controls and failed to differ statistically ( $p=0.303$ ;) among groups (Table 4).

The effect of the BB genotype on the occurrence of exudative AMD was estimated using a odds ratio estimate (OR) in case control studies. This OR was 3.079 (95% confidence interval [CI] 1.116 to 8.490) for the entire groups of patients and controls. When the male subgroups of cases and controls were tested separately, the effect was stronger with a OR of 5.276 (95% CI 1.267 to 21.961). For the female subgroup the OR was calculated to be 2.11 (95% CI 0.493 to 9.024). Data are shown in Table 5.

We performed Kaplan-Meier survival analysis on the data obtained from patients and control subjects. The average disease-free survival time was 83 years (95% CI=82-85; S.E.=1) in the pooled *CST3* AA or AB subjects and 75 years (95% CI=71-79; S.E.=2) in *CST3* BB subjects reaching statistical significance (Mantel-Cox Log rank=9.00; df=1;  $p=0.0027$ ).



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**CLAIMS**

1. A method for diagnosing or prognosing age-related macular degeneration in a subject, or determining whether a subject is at increased risk of developing age-related macular degeneration, comprising:  
determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in a sample from said subject;  
and  
comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status,  
thereby diagnosing or prognosing said age-related macular degeneration in said subject, or determining whether said subject is at increased risk of developing age-related macular degeneration.
2. The method according to claim 1, wherein said sample is taken from an eye of said subject, in particular from material located between the plasma membrane and basal lamina of the retinal pigment epithellum and/or from material located between the basal lamina of the retinal pigment epithelium and the inner collagenous zone of Bruch's membrane.
3. The method according to claim 2, wherein an increase of said level or activity of said substances in said sample from said subject relative to said reference value indicates a diagnosis, or prognosis, or increased risk of said age-related macular degeneration in said subject.

- 4. The method according to at least one of claims 1 to 3, wherein said subject is a human.
- 5. The method according to at least one of claims 1 to 4, wherein said cystatin C is determined in its monomeric form.
- 6. The method according to at least one of claims 1 to 5, wherein at least one of said substances is detected using an immunoassay, an enzyme activity assay and/or a binding assay.
- 7. The method according to at least one of claims 1 to 6, wherein said reference value is that of a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in a sample from a subject not suffering from said age-related macular degeneration.
- 8. A method of monitoring the progression of age-related macular degeneration in a subject, comprising:
  - determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in a sample from said subject;
  - and
  - comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status,

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thereby monitoring the progression of said age-related macular degeneration in said subject.

9. The method of claim 8, further comprising comparing a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in said sample with a level, an activity, or both said level level and said activity, of at least one of said substances in a series of samples taken from said subject over a period of time.
10. The method according to claim 8 and/or 9, wherein said subject receives a treatment prior to one or more of said sample gatherings.
11. A method of evaluating a treatment for age-related macular degeneration, comprising:
- determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for an amyloid protein, in a sample obtained from a subject being treated for said age-related macular degeneration;
- and
- comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status,
- thereby evaluating said treatment for said age-related macular degeneration.

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12. The method of claim 11, wherein said level, or said activity, or both said level and said activity, in said samples is determined, before and after said treatment is administered to said subject.
13. A method of diagnosing or prognosing age-related macular degeneration in a subject, or determining whether a subject is at increased risk of developing age-related macular degeneration comprising:  
determining a presence or absence of a mutation or polymorphism in a cystatin C gene or its non-coding regulatory elements in a sample from said subject,  
thereby diagnosing or prognosing age-related macular degeneration in said subject, or determining whether said subject is at increased risk of developing age-related macular degeneration.
14. The method of claim 13, wherein a presence of a mutation or polymorphism in leucin 88 codon of a human cystatin C gene leading to a loss of *Afu* I restriction site does not indicate diagnosis, or prognosis, or increased risk of age-related macular degeneration in said subject.
15. The method of claim 13 and/or 14, wherein the presence or absence of at least one B allele is determined.
16. The method of claim 15, wherein the presence of at least one B allele, in particular the presence of the B/B genotype, indicates said subject is at increased risk of developing age-related macular degeneration or indicates a diagnosis or prognosis of age-related macular degeneration.
17. The method of at least one of claims 13 to 16, further comprising:  
determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of

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cystatin C, a transcription product of a cystatin C gene, an amyloid protein, and a transcription product of a gene coding for an amyloid protein, in a sample from said subject;

and

comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status.

18. A kit for diagnosis, or prognosis, or determination of increased risk of developing age-related macular degeneration in a subject, said kit comprising:

- (a) at least one reagent which is selected from the group consisting of reagents that selectively detect cystatin C, reagents that selectively detect transcription products of a cystatin C gene, reagents that selectively detect an amyloid protein, reagents that selectively detect transcription products of a gene coding for an amyloid protein, and reagents that selectively detect a mutation or polymorphism in a cystatin C gene or its non-coding regulatory elements; and
- (b) instructions for diagnosing, or prognosing age-related macular degeneration, or determining increased risk of developing age-related macular degeneration by
  - (i) detecting a level, or an activity, or both said level and said activity, of said cystatin C, or of said transcription products of said cystatin C gene, or of said amyloid protein, or of said transcription products of said gene coding for an amyloid protein in a sample from said subject; and/or detecting a presence or absence of a mutation or polymorphism in said cystatin C gene or its non-coding regulatory elements in a sample from said subject; and

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(ii) diagnosing, or prognosing, or determining whether said subject is at increased risk of developing age-related macular degeneration, wherein

a varied level, or activity, or both said level and said activity, of said cystatin C, or of said transcription products of said cystatin C gene, or of said amyloid protein, or of said transcription products of a gene coding for an amyloid protein, compared to a reference value representing a known health status;

or a level, or activity, or both said level and said activity, of said cystatin C, or of said transcription products of said cystatin C gene, or of an amyloid protein, or of a transcription product of a gene coding for an amyloid protein similar or equal to a reference value representing a known disease status;

or the presence of a mutation or polymorphism in said cystatin C gene or its non-coding regulatory elements indicates a diagnosis, or prognosis, or increased risk of developing age-related macular degeneration.

19. The kit according to claim 18, wherein a presence of a mutation or polymorphism in leucin 68 codon of a human cystatin C gene leading to a loss of *A/u* I restriction site does not indicate diagnosis or prognosis of age-related macular degeneration in said subject.

20. The kit according to claim 18 and/or 19, wherein said reference value is that of a level, or an activity, or both said level and said activity, of at least one of said substances in a sample from a subject not suffering from said age-related macular degeneration.

21. The kit according to at least one of claims 18 to 20, wherein the presence of at least one B allele, in particular the presence of the B/B genotype, indicates

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a diagnosis, or prognosis, or an increased risk of development of age-related macular degeneration.

22. The kit according to at least one of claims 18 to 21 for use in monitoring a progression of age-related macular degeneration in a subject.
23. The kit according to at least one of claims 18 to 22 for use in monitoring success or failure of a therapeutic treatment of said subject.
24. A method of treating or preventing age-related macular degeneration in a subject comprising administering to said subject in a therapeutically effective amount an agent or agents which directly or indirectly affect an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein.
25. The method according to claim 24, wherein said agents are cathepsin derivatives or cystatin C analogs.
26. The method according to claim 24 and/or 25, wherein per se known methods of gene therapy and/or antisense nucleic acid technology are applied to administer said agent or agents.
27. The method according to at least one of claims 24 to 26 comprising grafting donor cells into the eye of said subject, said subject or donor cells preferably treated so as to minimize or reduce graft rejection, wherein said donor cells

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are genetically modified by insertion of at least one transgene encoding said agent or agents.

28. An agent which directly or indirectly affects an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein.
29. A medicament comprising an agent according to claim 28.
30. An agent which directly or indirectly affects an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein, for treating or preventing age-related macular degeneration.
31. Use of an agent which directly or indirectly affects an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, an amyloid protein, for a preparation of a medicament for treating or preventing age-related macular degeneration.



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32. A method for identifying an agent that directly or indirectly affects an activity, or level, or both said activity and level, of at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, cystatin C, a transcription product of a cystatin C gene, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein, comprising the steps of:

- (a) providing a sample containing at least one substance which is selected from the group consisting of a cystatin C gene, its non-coding regulatory elements, a transcription product of a cystatin C gene, cystatin C, a gene coding for an amyloid protein, its non-coding regulatory elements, a transcription product of a gene coding for an amyloid protein, and an amyloid protein;
- (b) contacting said sample with at least one agent;
- (c) comparing an activity, or level, or both said activity and level, of at least one of said substances before and after said contacting.

33. The method according to claim 32, wherein comparing an activity of cystatin C is performed by using amyloid precursor protein as a substrate and a generation of A-beta peptides as a read-out.

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**Abstract**

**A method for diagnosing or prognosing age-related macular degeneration in a subject, or determining whether a subject is at increased risk of developing age-related macular degeneration, comprising:**

**determining a level, or an activity, or both said level and said activity, of at least one substance which is selected from the group consisting of cystatin C, a transcription product of a cystatin C gene, an amyloid protein, a transcription product of a gene coding for an amyloid protein in a sample from said subject;**

**and**

**comparing said level, or said activity, or both said level and said activity, of at least one of said substances to a reference value representing a known disease or health status,**

**thereby diagnosing or prognosing said age-related macular degeneration in said subject, or determining whether said subject is at increased risk of developing age-related macular degeneration.**

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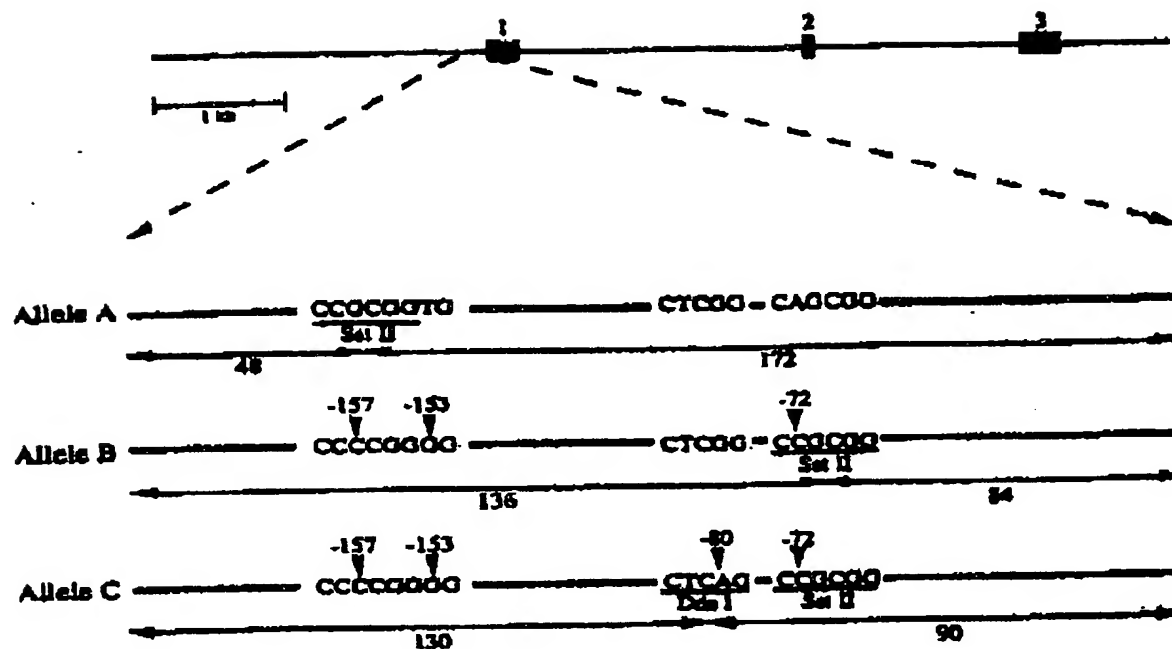


Figure 1

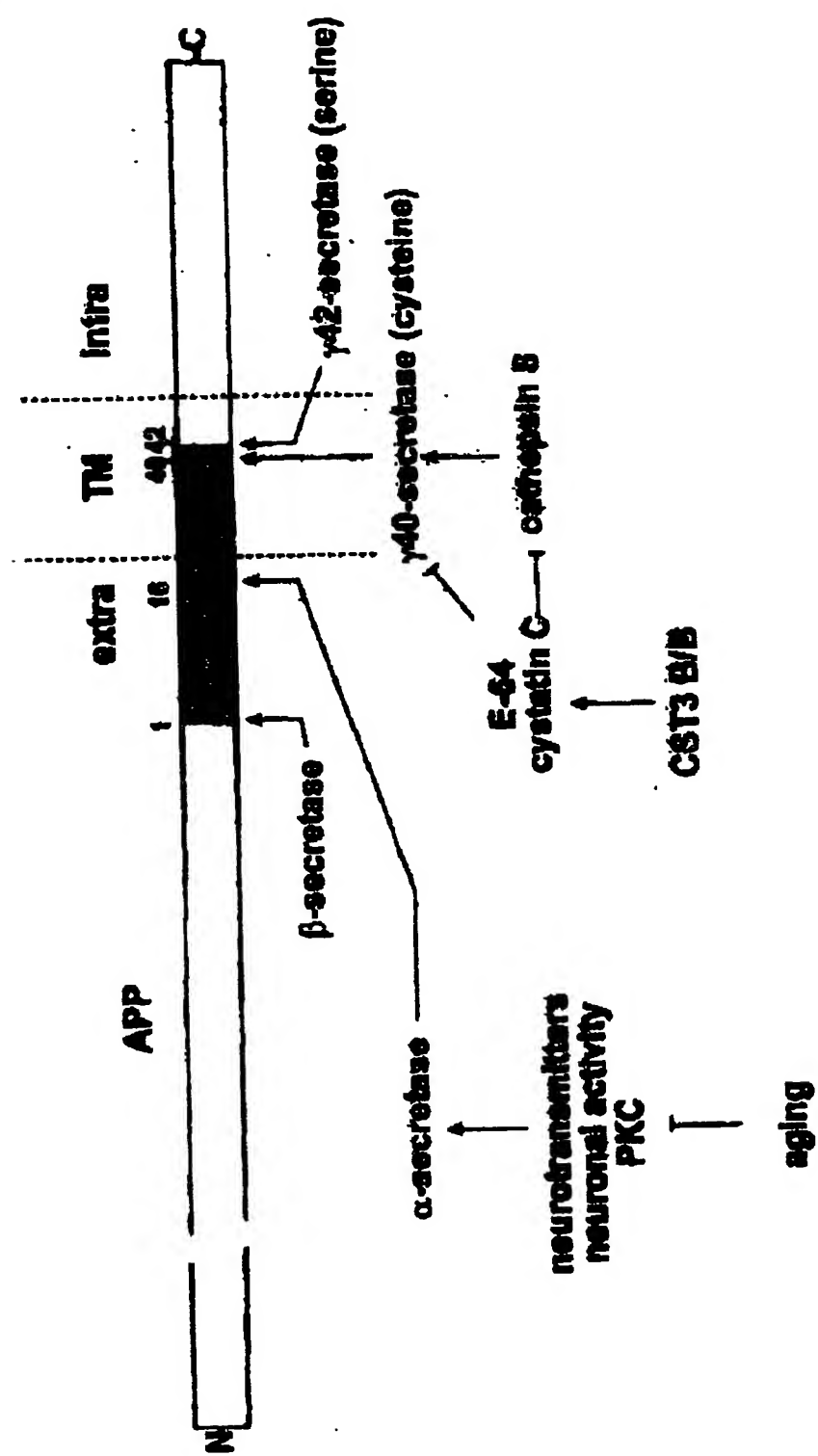


Figure 2

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**Table 1**

	Patients	Controls
<b>Sex (No.[%])</b>		
Female	114 (42.5)	141 (57.5)
Male	53 (31.7)	127 (68.3)
<b>mean age at presentation (yrs.±SD)</b>		
Female	75.25 ±7.63	75.30 ±7.89
Male	73.57 ±7.41	73.66 ±6.63

**Table 2**

CST3 genotype	Patients (n=167)		Controls (n=268)	
	n	%	n	%
AA or AB	156	93.4	262	97.8
BB	11	6.6	6	2.2

 $\chi^2=5.18$ ;  $p=0.0228$ ;  $df=1$ 
**Table 3**

CST3 genotype	Patients (n=53)		Controls (n=127)	
	n	%	n	%
AA or AB	47	88.7	124	97.6
BB	6	11.3	3	2.4

 $\chi^2=6.318$ ;  $p=0.0119$ ;  $df=1$ ; Fisher's exact test:  $p=0.0202$

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**Table 4**

CST3 genotype	Patients (n=114)		Controls (n=141)	
	n	%	n	%
AA or AB	109	95.6	138	97.9
BB	5	4.4	3	2.1

$\chi^2=1.056$ ;  $p=0.303$ ;  $df=1$

**Table 5**

Subgroup	Odds ratio	95% CI
All patients	3.079	1.117-8.49
Female	2.11	0.493-9.024
Male	5.276	1.267-21.961

Figure 4

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